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An anti-curare effect of hexamethonium at the mammalian neuromuscular junction

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Summary

1. Experiments were performed on the isolated phrenic nerve and diaphragm preparation of the rat.

2. In preparations partly blocked with (+)-tubocurarine, the twitch amplitude increased after hexamethonium. This enhancement was not seen in preparations partly blocked with Mg^{++} or with gallamine. High concentrations of hexamethonium produced failure of contraction.

3. Extracellular endplate potentials were recorded from blocked preparations. The administration of hexamethonium resulted in an increased amplitude of these potentials only in curarized muscle.

4. Hexamethonium had no anticholinesterase activity nor did it depolarize muscle cells or increase the quantal release of transmitter.

5. It is concluded that hexamethonium exerts a specific anti-curare action. Experiments on the recovery of the twitch after washing out antagonists indicate that this process is limited by diffusion. The difference in rates of diffusion of hexamethonium and (+)-tubocurarine does not account for their interaction. The basis of the anti-curare action of hexamethonium is discussed.

Introduction

As part of a course of experiments in pharmacology for undergraduates, the effects of antihypertensive drugs were studied on the pithed rat preparation (Gillespie & Muir, 1967). In this preparation, the sympathetic outflow was stimulated through the steel needle used to pith the rat; twitching of the skeletal muscles, due to spread of the stimulating current to the motor axons, was prevented by blocking neuromuscular transmission with (+)-tubocurarine chloride (1 mg/kg) given intravenously. It was noticed that after intravenous injection of hexamethonium bromide, 10 mg/kg, the twitches of the skeletal muscles returned. Therefore, the possibility of an interaction between hexamethonium and (+)-tubocurarine was investigated *in vitro*; a preliminary account of our findings has been communicated to the British Pharmacological Society (Ferry & Marshall, 1971).

Methods

The phrenic nerves and diaphragms were dissected from white rats, each weighing about 200 g and of either sex, which had been killed by a blow on the head and section of the spinal cord in the neck. Both right and left hemidiaphragms were used and were bathed in medium of the following composition (mM): NaCl, 137; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; NaH_2PO_4, 1; $NaHCO_3$, 12; dextrose, 25; and aerated with

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5% CO_2 in O_2. For electrical recording, the diaphragm was pinned down in a Perspex bath and illuminated from below. Medium flowed into the bath at approximately 1 ml/min and the amount in the bath was kept at 20 ml by a constant level device and at 37° C. The phrenic nerve was stimulated through a pair of silver electrodes mounted in a perspex tube.

Intracellular recordings were made with glass microelectrodes filled with 3 M KCl, resistance 10–20 MΩ. Extracellular records were made with insulated silver wire electrodes (0·13 mm diam., diamel coated ; Johnson, Matthey & Co.), suitably supported in glass capillary tubing. One electrode was located at the endplate region, the indifferent electrode being placed in the medium. The endplate region was located in blocked preparations by positioning the electrode so that the amplitude of the endplate potential and its rate of rise were maximal and a presynaptic spike was seen. The recording system had a bandwidth of 10 KHz to 0·2 Hz (−3 db points).

The contractile response of the diaphragm was recorded with a Starling's heart lever writing on a smoked drum, after stimulation of the phrenic nerve at 0·1 Hz. In later experiments an isometric strain gauge (Devices UFl) was used with a Devices M2 recorder.

Determination of acetylcholinesterase activity

The activity of the acetylcholinesterase of erythrocytes was determined by a titrimetric method using a Radiometer pH-stat auto titration unit. The enzyme was prepared by washing human erythrocytes (prepared from bank blood) five times with distilled water and once with saline, and finally suspending the packed ghosts in nine volumes of saline. The saline used had a composition similar to that of the medium given above, but lacked $NaHCO_3$, NaH_2PO_4 and dextrose and was not gassed. For estimations of enzyme activity, 0·5 ml of the enzyme preparation was diluted to a final volume of 6 ml in a reaction vessel and was pre-incubated at 37° C for 10 minutes. The pH was adjusted to 7·4 and the reaction started by addition of a concentrated solution of acetylcholine perchlorate from a microsyringe to give a final concentration of 1 mM. The titrator recorded the time course of the addition of 5 mM NaOH which maintained the pH at 7·4 by neutralizing the acetic acid produced during the hydrolysis of acetylcholine. The initial rate of acetylcholine hydrolysis was determined from the slope of this record. Corrections were made for the spontaneous hydrolysis of acetylcholine and for the absorption of atmospheric CO_2.

Drugs

The drugs used were hexamethonium bromide (May & Baker Ltd.) and (+)-tubocurarine chloride (Burroughs Wellcome).

Results

The effect of hexamethonium on muscle contractions

In 15 experiments, a phrenic nerve–diaphragm preparation was immersed in medium and the contractions were recorded after stimulation of the nerve at 0·1 Hz. A partial block of neuromuscular transmission was produced by the addition of (+)-tubocurarine in concentrations ranging from 0·3–1·5 μM, a steady state

being established in about 30 minutes. Hexamethonium was then added in a concentration of 14–400 μM and an increased twitch was seen within 5 min in all experiments; a near maximal enhancement of the twitch was seen with 300 μM hexamethonium, and this concentration was used in most subsequent experiments. The degree of enhancement of the twitch was variable, and depended, in part, on the initial depth of the block. Figure 1 shows the results of one experiment in which hexamethonium had a considerable effect on an almost completely blocked preparation. The enhanced response after the addition of hexamethonium usually did not exceed 50% of the initial unblocked twitch. When hexamethonium was washed out with the solution of (+)-tubocurarine, the twitch decreased in amplitude.

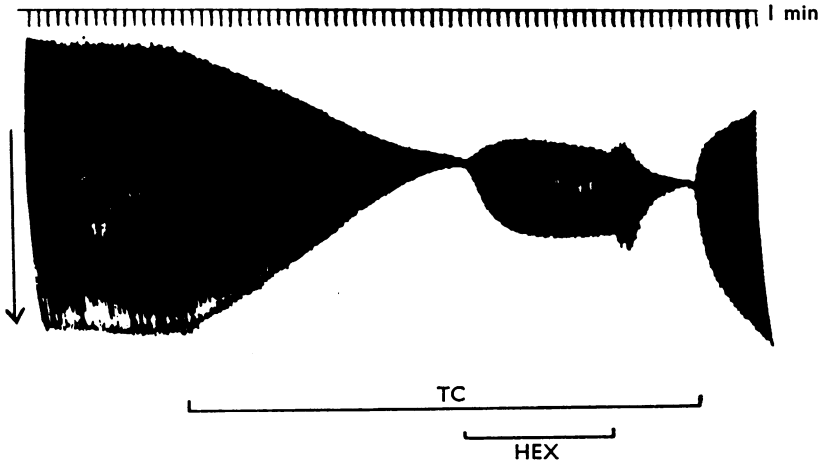

FIG. 1. Rat phrenic nerve diaphragm *in vitro*. 37° C. Contractile response to nerve stimulation at 0·1 Hz recorded with a heart lever. The tracing shows the onset of almost complete block after (+)-tubocurarine 1·3 μM (TC) and the enhancement of the twitch after hexamethonium 360 μM (HEX). The enhancement diminished after washing out the hexamethonium.

In some experiments, a partial block was produced by gallamine 170 μM, and in these experiments the addition of hexamethonium did not result in an increased twitch, merely an intensification of the block. In a similar type of experiment, partial block was produced by 6 mM Mg^{++}; after the addition of hexamethonium the twitch became smaller.

In unblocked preparations, the addition of increasing doses of hexamethonium had no apparent effect until a concentration of 2 mM was reached when a 10% reduction of the twitch amplitude was seen. In the presence of 4 mM hexamethonium there was complete failure of the contraction after stimulation. In a preparation partially blocked with (+)-tubocurarine, the response was further depressed after the addition of hexamethonium, 0·7 mM.

These experiments show that hexamethonium can increase the contractile response of partly blocked muscle to nerve stimulation. This facilitatory effect is obtained only in preparations blocked by (+)-tubocurarine. Hexamethonium in large doses blocks the response to nerve stimulation and acts synergistically with (+)-tubocurarine.

The effect of hexamethonium on extracellularly recorded endplate potentials

In four preparations, neuromuscular transmission was blocked after incubation for 1 h in (+)-tubocurarine 1·8 μM. The currents flowing in the external medium were recorded with a wire electrode located at the endplate region. The records show the stimulus artefact followed by small positive and negative deflexions attributable to the action potential in the nerve terminals, followed by a much larger negative-going potential attributable to current flowing into the endplate. This extracellular endplate potential (e.e.p.p.) was evoked and recorded at 0·1 Hz or 1 Hz. In most experiments, incremental concentrations of hexamethonium were applied to the diaphragm, and records of the response made 10 min after each increment; the concentrations used ranged from 2·7 μM to 600 μM. In all experiments, the amplitude of the e.e.p.p. increased with low concentrations of hexamethonium, but after higher concentrations it decreased towards the control size. Records from an experiment which show the presynaptic and postsynaptic currents and the effect of hexamethonium are depicted in Figure 2. The results from 4

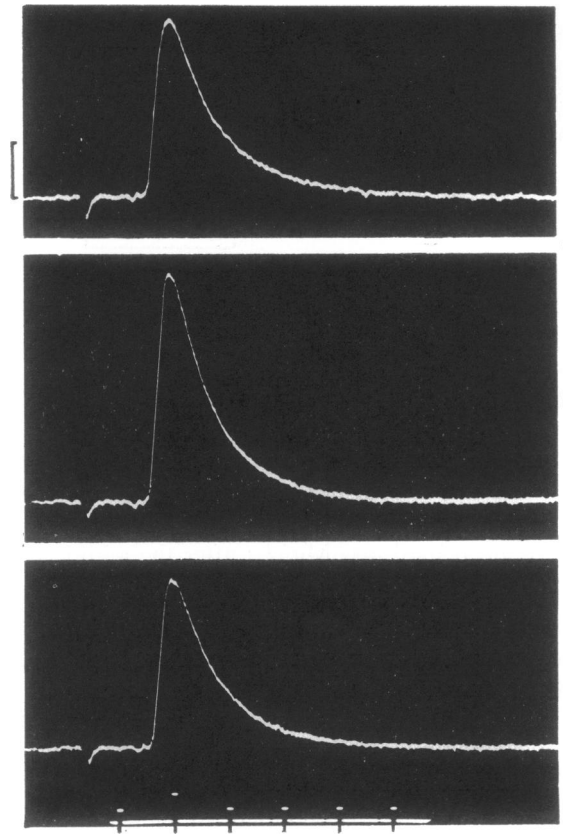

FIG. 2. Rat phrenic nerve diaphragm *in vitro*. 37° C. (+)-Tubocurarine 1·8 μM. Records of the endplate potential recorded with extracellular electrodes. Note small presynaptic potential. a. Control record. b. After hexamethonium 280 μM. Note increase of the amplitude to 1·3 times the control. c. After hexamethonium 550 μM. Amplitude 0·97 times control. Calibration 0·1 mV. Time scale 1 ms.

preparations are shown in Fig. 3 which clearly demonstrates the biphasic effect of hexamethonium on e.e.p.p. amplitude in preparations blocked by (+)-tubocurarine. When transmission was blocked by gallamine 220 μM, the addition of hexamethonium resulted in depression of the e.e.p.p. amplitude. Similar experiments were made in preparations blocked with Mg^{++} 12 mM; in these experiments, because the amplitude of the e.e.p.p. was variable due to variations in quantal release of acetylcholine, the mean of 8 responses was used as a measure of e.e.p.p. amplitude. After hexamethonium, the mean amplitude of the e.e.p.p. was decreased. The results of the experiments with gallamine and with Mg^{++} are also shown in Figure 3.

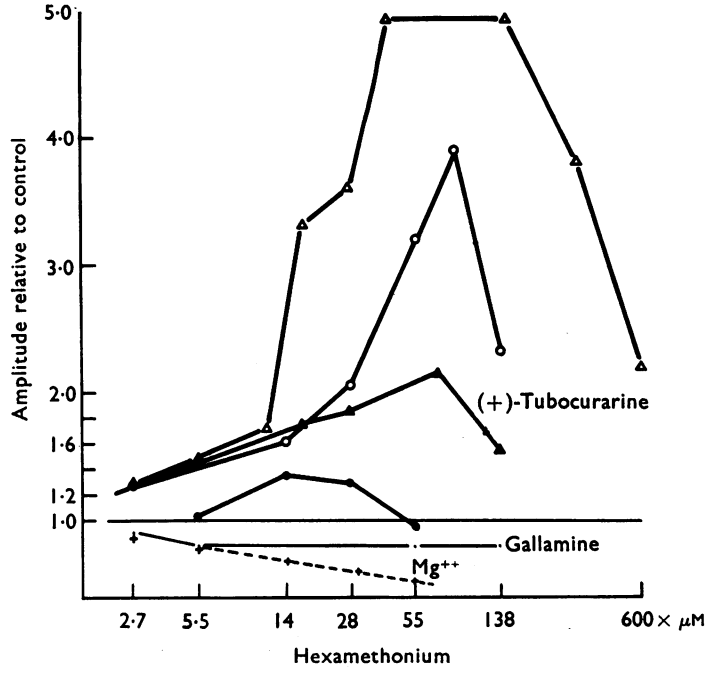

FIG. 3. Graph showing the relation between the concentration of hexamethonium and the enhancement of the amplitude of the extracellularly recorded endplate potential in six phrenic nerve diaphragm preparations. Four of these had been blocked with (+)-tubocurarine 1·8 μM, and hexamethonium always increased the amplitude of the response. In each of the diaphragms blocked with gallamine 220 μM or with Mg^{++} 10 mM, hexamethonium decreased the response.

In addition to an increase in the amplitude of the e.e.p.p. after the addition of hexamethonium, there were changes in its time course, represented by its duration at half amplitude. After hexamethonium, the duration of the e.e.p.p. at half amplitude was sometimes increased and sometimes decreased. The mean value of the duration of e.e.p.p.s, relative to their control in (+)-tubocurarine, was 1·06 (S.E. 0·14) after the administration of hexamethonium in 4 experiments; and the mean amplitude relative to control was 3·1 (S.E. 0·8). It is known that anti-cholinesterase drugs increase both the amplitude and duration of the e.e.p.p. to a similar extent (Ferry & Marshall, unpublished results) and so the relatively small and inconsistent effect of hexamethonium on the duration of the e.e.p.p. suggests that an inhibition of cholinesterase is not the cause of the increase in the amplitude of the e.e.p.p.

The effect of hexamethonium on acetylcholinesterase activity

The rate of hydrolysis of 1 mM acetylcholine by a preparation of erythrocyte ghosts was determined by a titrimetric method at 37° C and pH 7·4. The initial rate of hydrolysis of acetylcholine was determined in the absence of drugs, and also with (+)-tubocurarine 2·6 μM, or with hexamethonium 280 μM, or with both (+)-tubocurarine and hexamethonium. The results of two similar experiments are shown in Table 1. It can be seen that hexamethonium alone did not reduce the rate of hydrolysis of acetylcholine. (+)-Tubocurarine caused a 15% reduction in the rate of acetylcholine hydrolysis and no further reduction was seen when the medium contained both (+)-tubocurarine and hexamethonium. It is concluded that (+)-tubocurarine has an inhibitory action on the acetylcholinesterase of human erythrocyte ghosts and that hexamethonium has no such action.

TABLE 1. *The results of two experiments on the effect of* (+)*-tubocurarine and hexamethonium on the velocity of hydrolysis of acetylcholine,* 1 μM *by erythrocyte ghosts at* 37° *C and pH* 7·4

Drug	Rate of acetylcholine hydrolysis n moles/s		Relative rate
None	1·55 1·53	1·54	1
(+)-Tubocurarine 2·6 μM	1·32 1·31	1·32	0·86
Hexamethonium 280 μM	1·59 1·50	1·55	1
(+)-Tubocurarine + hexamethonium	1·32 1·31	1·32	0·86

The effect of hexamethonium on the release of transmitter

Because some anti-curare drugs exert their effect by increasing the number of quanta released by nerve impulses, experiments were made to investigate the possibility of such an action of hexamethonium.

Phrenic nerve–diaphragm preparations were blocked with (+)-tubocurarine 1·6 μM. Endplate potentials were elicited at 1 Hz and were recorded with an intracellular microelectrode inserted into a muscle cell at the endplate. The amplitudes of a series of about 30 endplate potentials were measured, and the mean quantal content of the endplate potential calculated by analysis of the variance of the potentials. Since there is a considerable variation of the mean quantal content between endplates, the overall mean quantal content was determined from a number of junctions. Under control conditions, the overall mean quantal content of the endplate potential determined at 31 endplates in 4 preparations was 178 (S.E. 13). In a further 2 preparations, after the addition of hexamethonium 280 μM, the overall mean quantal content of the endplate potential determined at 34 endplates was 149 (S.E. 12). This value is not significantly different from that of the control ($P=0·1$).

It is concluded that the increased e.e.p.p. after hexamethonium is not associated with an increased quantal release of transmitter.

The effect of hexamethonium on the membrane potential

In one preparation treated with (+)-tubocurarine 1·8 μM, the membrane potential at the endplate region was measured. The mean value of 31 cells was 71·3 mV

(S.E. 0·74 mV). Hexamethonium 280 μM was then added and the mean membrane potential of another 21 cells determined and found to be 71 mV (S.E. 0·6 mV). It is concluded that hexamethonium had no depolarizing effect under these conditions ($P=0·8$). It would appear that hexamethonium does not facilitate neuromuscular transmission by any of the 'conventional' actions, and thus some other action must be sought.

Experiments on the frog rectus preparation

The possibility that hexamethonium interferes with the blocking action of (+)-tubocurarine at cholinergic receptors was investigated by experimenting on the frog rectus. Contractures in response to the application of acetylcholine for 2 min were recorded in the presence of (+)-tubocurarine 0·57 μM and then again after the addition of hexamethonium 280 μM. It was found that, after hexamethonium, the response to acetylcholine was not increased. Thus no anti-curare action of hexamethonium was demonstrated.

It may be that the short duration of transmitter action at the phrenic neuromuscular junction compared with the 2 min exposure to acetylcholine in the rectus is a crucial factor for the production of the anticurare effect of hexamethonium. There is some precedent for this suggestion, since an evanescent action of an agonist was suggested by Paton & Waud (1967) to account for an apparently non-competitive action of (+)-tubocurarine. It has also been shown that potentiation of agonist action in the presence of an antagonist by a second antagonist can occur, and this phenomenon depends on a short duration of agonist action (Stephenson & Ginsborg, 1969).

Experiments on the wash-out time of blocking agents

The work of Paton & Waud (1967) and of Stephenson & Ginsborg (1969) affords an explanation of the anti-curare action of hexamethonium. In a preparation blocked with (+)-tubocurarine, the duration of transmitter action, about 4 ms, may be too short to permit much progress towards a new equilibrium between the receptors, the transmitter and the blocker, and so (+)-tubocurarine would act effectively as a non-competitive antagonist at the receptors. During the 10 min allowed to elapse after adding hexamethonium, a new equilibrium may be set up between the receptors, hexamethonium and (+)-tubocurarine. After the release of transmitter, (+)-tubocurarine would remain effectively non-competitive, but perhaps there may be some movement towards a new equilibrium between the receptors, the transmitter and hexamethonium which would result in a greater agonist action. On this hypothesis of Stephenson & Ginsborg (1969), the important difference between hexamethonium and (+)-tubocurarine which is the basis of the interaction, is the rate at which the drug can leave the receptors. Experiments were therefore made on the rate of recovery of the twitch of phrenic nerve diaphragm preparations completely blocked by a blocking agent in an attempt to estimate the rate of clearance of this drug from the receptors.

Preparations were blocked initially with a high concentration of hexamethonium, (+)-tubocurarine or gallamine and the drug was then washed out and the preparation allowed to recover. A second dose of hexamethonium (5 mM) or (+)-tubocurarine (1 μM) or gallamine (200 μM) was added and the latency of the onset of complete block was measured. The drug was allowed to remain in the bath for a

TABLE 2. *The results of experiments in which nerve-diaphragm preparations were completely blocked and the time to half recovery determined after washing out the drug*

Drug	Number of preparations	Number of determinations	Mean half-recovery time (min)	S.E.
(+)-Tubocurarine 1 μM	4	9	3·9	0·3
Hexamethonium 5 mM	4	12	1·2	0·1
Gallamine 200 μM	4	10	2·1	0·2

further period equal to this latency and was then washed out. The time taken for the twitch, elicited at 0·1 Hz, to recover to 0·5 of the control was determined. These half-recovery times are shown in Table 2. It can be seen that the half-recovery time for (+)-tubocurarine is 3·25 times that of hexamethonium. In one preparation a crossover experiment was made in which the half-recovery time was determined first for hexamethonium and then for (+)-tubocurarine and finally for hexamethonium again; and in another experiment, (+)-tubocurarine was given before and after hexamethonium. In these experiments the half-recovery time for (+)-tubocurarine was longer than for hexamethonium by a factor of about 2·3.

These results show that the blocking action of (+)-tubocurarine persists at the endplate longer than that of hexamethonium.

Discussion

Half-recovery times

The increase in the amplitude of the twitch after washing out an antagonist represents an increase in the number of junctions at which the block has declined sufficiently to allow transmission to occur. The proportion of receptors cleared during this time may be estimated from the curves which relate the receptor occupancy by an antagonist with the amplitude of the twitch (Paton & Waud, 1967; Barnard, Wiekowski & Chiu, 1971). Data from these curves are shown in Table 3. From the fractional occupancy at complete block and at half block of the contractions and the half-recovery time, the time constant of receptor clearance may be calculated. The minimum values of this, which are derived from the data of Barnard *et al.* (1971) are 4·4 min for hexamethonium and 14·4 min for (+)-tubocurarine. Thus, during the 4 ms of transmitter action, there would be very little clearance of (+)-tubocurarine or of hexamethonium from the receptors, and both blocking agents would, on this basis, be effectively non-competitive antagonists. If this is so there could be no anti-curare action of hexamethonium like that suggested by Stephenson & Ginsborg (1969). However, it may be that the time constant of receptor clearance calculated from our results does not measure the rate of dissociation of the antagonist-receptor complex; indeed, Brooks & MacKay (1971a) have suggested that the recovery time is determined by the rate of diffusion of the drug from the synapse.

TABLE 3. *The relation between the amplitude of the twitch and the fractional occupancy of receptors by an inhibitor taken from Paton & Waud* (1967) *and Barnard* et al. (1971)

Contraction relative to control	Occupancy of antagonist	
	Paton & Waud	Barnard *et al.*
0	>0·917	0·904
0·5	0·838	0·69
0·03	0·917	0·857
0·35	0·871	0·738

The twitch amplitude~occupancy curves suggest that if a just-adequate concentration of antagonist were used to achieve complete block of transmission, after washing out the antagonist from the bath the concentration in the biophase would fall to between one-half and one-quarter of the initial concentration at the half-recovery time. Thus the concentrations of different drugs would fall by the same relative amount and as the dimensions of the synaptic cleft are likely to be similar in different diaphragms, so the half-recovery time should be a measure of the reciprocal of the diffusion coefficient. As the diffusion coefficient is also proportional to the reciprocal of the root of the molecular weight, then the diffusion coefficient calculated from the half-recovery time may be compared with that calculated from the molecular weight.

Antagonist	Molecular weight	(Molecular weight)$^{-0.5}$	Half-recovery time (min)	(Half-recovery time)$^{-1}$
Hexamethonium	102·4	0·099 (2·25)	1·2	0·833 (1·75)
Gallamine	510·8	0·044 (1·00)	2·1	0·476 (1·00)
(+)-Tubocurarine	624·8	0·040 (0·91)	3·9	0·256 (0·54)

The diffusion coefficients calculated relative to that of gallamine are shown in parentheses. The diffusion coefficient of (+)-tubocurarine relative to gallamine when determined from conductivity measurements (Brooks & MacKay, 1971b) varied from 0·61 to 1·03. Because of the similarities in the diffusion coefficients of hexamethonium and (+)-tubocurarine relative to that of gallamine when calculated from the molecular weight and from the experimentally determined half-recovery time, we conclude that the time constant of clearance of the receptors when the antagonists were washed out was determined by the diffusion of the drug from the synaptic cleft, and this confirms the conclusion of Brooks & MacKay. It is clear that the difference in rate of diffusion of (+)-tubocurarine and hexamethonium does not account for their interaction, for receptor clearance is too slow. The possibility remains that the interaction could be due to differences in the rate of dissociation of the antagonists from the receptors as suggested by Stephenson & Ginsborg (1969).

The anti-curare action of hexamethonium

Hexamethonium does not depolarize the endplate region, nor does it increase the quantal release of transmitter or prolong the duration of action by increasing the membrane resistance or by acting at some point between the activation of receptors and the increase in membrane permeability, because it would be expected that such actions would have facilitated transmission in preparations blocked by gallamine or by Mg^{++}. The anti-curare action of hexamethonium and the lack of facilitation in preparations blocked with Mg^{++} are explicable by the hypothesis of Stephenson & Ginsborg (1969). In the case of Mg^{++}, the fractional occupancy by acetylcholine would be reduced following a diminished quantal release. Attempts to show differences in the rate of dissociation of hexamethonium and (+)-tubocurarine from the receptors, which would be the basis of the interaction, have been unsuccessful. However, Stephenson & Ginsborg (1969) provided a mathematical model of the potentiation by an antagonist, and this can be applied to our results.

From Stephenson & Ginsborg (1969):

$$\frac{y_{bc}}{y_b} = \frac{1 - X}{(1 - XY)(1 - XZ)}$$

where y_{bc} is the fraction of receptors occupied by the agonist in the presence of both

slowly dissociating (b) and rapidly dissociating (c) antagonists; y_b is the fraction occupied by the agonist in the presence of only b; X is the fraction of receptors which would have been occupied by c, Y by the agonist and Z by b at equilibrium if each of these substances had been present alone.

In the experiment shown in Fig. 1, after (+)-tubocurarine the response decreased to 0·03 of the control. After adding hexamethonium the twitch increased to 0·35.

The ratio $\frac{y_{bc}}{y_b}$ can be estimated from these experimental results using the curves of Paton & Waud (1967) and Barnard *et al.* (1971) to give the appropriate values of the fractional occupancy by the antagonists (see Table 3). Assuming that receptor occupation is complete, then these values represent $(1-y_b)$ and $(1-y_{bc})$. On this basis, the values of $\frac{y_{bc}}{y_b}$ calculated from the data of Paton & Waud and Barnard *et al.* are 1·55 and 1·83 respectively.

These values may be compared with those derived from the equation of Stephenson & Ginsborg. Estimates must be made of the variables X, Y and Z. The value of Z was assumed to by $1-y_b$. The fractional occupancy of 360 μM hexamethonium alone, X, can be derived by the Langmuir equation from the concentration which caused complete block, 3 mM, and the occupancy which is associated with this taken from the data of Paton & Waud and Barnard *et al.* The values of X are 0·52 and 0·48 respectively. The remaining quantity to be included is Y, the fractional occupancy of the agonist alone. Negrete, Del Castillo, Escobar & Yanklevich (1972) have suggested that receptors at the endplate are saturated with transmitter released from the nerves, thus a value of 0·99 might be appropriate for Y.

The value of the right hand side of the above equation is 1·89 using the data of Paton & Waud, and 1·68 using the data of Barnard *et al.* The close similarity between the values of $\frac{y_{bc}}{y_b}$ calculated from the experimental results and those calculated from estimations of X, Y and Z suggests that the hypothesis of Stephenson & Ginsborg can be invoked to explain the anti-curare action of hexamethonium. This being so, the time constant for the dissociation of hexamethonium from the receptors should be about 2 ms, and for (+)-tubocurarine, about 40 milliseconds.

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